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The High Prevalence and Diversity of *Chlamydiales* DNA within *Ixodes ricinus* Ticks Suggest a Role for Ticks as Reservoirs and Vectors of *Chlamydia*-Related Bacteria

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The *Chlamydiales* order is composed of nine families of strictly intracellular bacteria. Among them, *Chlamydia trachomatis*, *C. pneumoniae*, and *C. psittaci* are established human pathogens, whereas *Waddlia chondrophila* and *Parachlamydia acanthamoebae* have emerged as new pathogens in humans. However, despite their medical importance, their biodiversity and ecology remain to be studied. Even if arthropods and, particularly, ticks are well known to be vectors of numerous infectious agents such as viruses and bacteria, virtually nothing is known about ticks and chlamydia. This study investigated the prevalence of *Chlamydiae* in ticks. Specifically, 62,889 *Ixodes ricinus* ticks, consolidated into 8,534 pools, were sampled in 172 collection sites throughout Switzerland and were investigated using pan-*Chlamydiales* quantitative PCR (qPCR) for the presence of *Chlamydiales* DNA. Among the pools, 543 (6.4%) gave positive results and the estimated prevalence in individual ticks was 0.89%. Among those pools with positive results, we obtained 16S rRNA sequences for 359 samples, allowing classification of *Chlamydiales* DNA at the family level. A high level of biodiversity was observed, since six of the nine families belonging to the *Chlamydiales* order were detected. Those most common were *Parachlamydiaceae* (33.1%) and *Rhabdochlamydiaceae* (29.2%). "Unclassified *Chlamydiales*" (31.8%) were also often detected. Thanks to the huge amount of *Chlamydiales* DNA recovered from ticks, this report opens up new perspectives on further work focusing on whole-genome sequencing to increase our knowledge about *Chlamydiales* biodiversity. This report of an epidemiological study also demonstrates the presence of *Chlamydia*-related bacteria within *Ixodes ricinus* ticks and suggests a role for ticks in the transmission of and as a reservoir for these emerging pathogenic *Chlamydia*-related bacteria.

Members of the *Chlamydiales* order are obligate intracellular bacteria, sharing a characteristic developmental cycle with elementary bodies (EBs) that are metabolically inactive and reticulate bodies (RBs) that are metabolically active within a host-derived replicative niche called "inclusion" (1). From 1907, when *Chlamydia trachomatis* was discovered by Halberstädter and Prowazek, to the 1990s, *Chlamydiales* order diversity was limited to a small group of closely related intracellular bacterial pathogens. During the last 20 years, knowledge of chlamydial biodiversity dramatically increased with the discovery of *Chlamydia*-related bacteria. Considering the new taxonomy rules (2, 3), in 2 decades, the *Chlamydiales* order moved from 1 to 9 family-level lineages: *Chlamydiaceae*, *Clavichlamydiaceae*, *Criblamydiaceae*, *Piscichlamydiaceae*, *Parachlamydiaceae*, *Rhabdochlamydiaceae*, *Simkaniaceae*, *Waddliaceae*, and "*Candidatus* Parilichlamydiaceae" (2–5). Bacteria of these families have been isolated or identified within a wide host range, and their diversity is certainly underestimated.

Nowadays, members of *Chlamydiales* are recognized to be among the most important human and animal pathogens. Among the *Chlamydiaceae* family, in addition to *Chlamydia trachomatis*, which is responsible for trachoma and genital tract infection, *C. pneumoniae* and *C. psittaci* are also well known to induce respiratory tract infections in humans (6, 7). Other species of the *Chlamydiaceae* family such as *C. abortus* are mainly of veterinary importance, being mainly involved in animal abortions. Most of these species are also able to cross the interspecies barrier, to infect humans, and to cause zoonotic infections affecting the human lungs and leading to miscarriage (8). Moreover, several *Chlamydia*-related bacteria such as *Parachlamydiaceae*, *Waddliaceae*, and *Simkaniaceae* spp. are increasingly recognized as emerging pathogens of veterinary and human medical importance with roles in bovine abortions (9, 10) and with significant implications in human respiratory tract infections and adverse pregnancy outcomes (11–13).

Regarding *Chlamydiales* ecology, these bacteria are extremely widespread around the world, with a huge range of nonhuman hosts, including vertebrates, such as mammals, marsupials, fish, amphibians, birds, and reptiles, as well as invertebrates such as insects, molluscs, crustaceans, and protozoa (14–17). This peculiar ability of most *Chlamydia*-related bacteria to grow within protozoa has been used to successfully isolate novel *Chlamydiae* by using amoebae in an innovative cell culture system called amoebal coculture (18, 19).

Received 3 July 2015 **Accepted** 15 September 2015

Accepted manuscript posted online 18 September 2015

Citation Pilloux L, Aeby S, Gaümann R, Burri C, Beuret C, Greub G. 2015. The high prevalence and diversity of *Chlamydiales* DNA within *Ixodes ricinus* ticks suggest a role for ticks as reservoirs and vectors of *Chlamydia*-related bacteria. Appl Environ Microbiol 81:8177–8182. doi:10.1128/AEM.02183-15.

Editor: H. L. Drake

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02183-15.

TABLE 1 Prevalence of *Chlamydiales* DNA within *Ixodes ricinus* ticks collected throughout Switzerland[a]

Sample category	No. of ticks	No. of pools	Pool prevalence (%) (no. of pools with *Chlamydiales* DNA/total no. of pools)	Estimated prevalence in individual ticks (%)	Maximum infection rate (%) (no. of ticks with *Chlamydiales* DNA/total no. of ticks)	Minimum infection rate (%)
Nymphs	42,576	4,331	6.9 (298/4,331)	0.72	6.9 (2,935/42,576)	0.7 (298/42,576)
Adults	20,313	4,203	5.8 (245/4,203)	1.24	5.9 (1,200/20,313)	1.2 (245/20,313)
Total	62,889	8,534	6.4 (543/8,534)	0.89	6.6 (4,135/62,889)	0.86 (543/62,889)

[a] The pool prevalence represents the number of positive pools compared to the total number of tested pools. The maximum prevalence, which was close to the pool prevalence, was calculated on the basis of the assumption that each individual tick of a positive pool was infected. The minimum prevalence was calculated on the basis of the assumption that only one individual tick of a positive pool is infected.

Some members of the *Chlamydiaceae* family have been documented to be transmitted by arthropods. *C. trachomatis* may be transmitted by flies (20), whereas *C. psittaci* may be transmitted by flies, mites, lice, and ticks (21–23). More recently, *Chlamydia*-related bacteria belonging to the *Simkaniaceae* and the *Rhabdochlamydiaceae* families have been isolated from arthropods (24–26). Moreover, very recently, a study highlighted the presence of *Chlamydiales* DNA in ticks and fleas (17). Since *Ixodes ricinus* ticks are among the most important vectors for animal- and human-pathogenic bacteria (27), this study was a detailed investigation of the prevalence and biodiversity of *Chlamydiales* DNA in more than 60,000 *Ixodes ricinus* ticks.

MATERIALS AND METHODS

Tick sampling, tick pooling, and DNA extraction. A total of 62,889 *Ixodes ricinus* ticks were collected by flagging low vegetation at 172 different sites throughout Switzerland (28). Ticks were identified by morphological characteristics as adults or nymphs and were pooled in groups ranging in size from 1 to 10 individuals. After tick homogenization, nucleic acids were extracted using an automated nucleic acid extraction system (QIAsymphony SP; Qiagen, Hilden, Germany) as described by Gäumann et al. (28). Extracted DNAs were then stored in 96-well microplates at −80°C until further use.

Pan-*Chlamydiales* real-time qPCR assay. An automated liquid handling workstation equipped with eight nondisposable fixed tips (Tecan EVO; Tecan, Männedorf, Switzerland) was used to transfer DNA extractions from 96-well microplates to 384-well microplates and to perform the quantitative PCR (qPCR) analyses. This qPCR was used to detect and amplify a fragment length of 207 to 215 bp of the 16S rRNA-encoding gene of *Chlamydiales*. Quantification was performed using a plasmidic 10-fold-diluted positive control tested in duplicate. Specifically, amplification reactions were performed in a final volume of 20 μl containing iTaq Universal Probes Supermix with ROX (Bio-Rad, Reinach, Switzerland), a 0.1 μM (each) concentration of primers panCh16F2 (5′-CCGC CAACACTGGGACT-3′) and panCh16R2 (5′-GGAGTTAGCCGGTGC TTCTTTAC-3′) (Eurogentec, Seraing, Belgium), a 0.1 μM concentration of probe panCh16S (5′-FAM [6-carboxyfluorescein]-CTACGGGAGGC TGCAGTCGAGAATC-BHQ1 [black hole quencher 1]-3′) (Eurogentec), molecular-biology-grade water (Five Prime, Hilden, Germany), and 5 μl of DNA sample. Underlined bases represent locked nucleic acids (LNA) (29). In each 384-well microplate, 16 wells were dedicated to negative controls (water), 12 wells were used as nucleic acid extraction negative controls, and 12 wells were used as positive controls, as described by Croxatto et al. (17). Amplification started with an initial step of activation and denaturation at 95°C for 3 min that was followed by 50 cycles at 95°C for 15 s, 67°C for 15 s, and 72°C for 15 s and was performed in a 7900 HT Fast real-time PCR system (Applied Biosystems, Zug, Switzerland). Samples with a threshold cycle (C_T) value of >37 were considered to have given negative results.

DNA sequencing of the qPCR-positive samples. Amplicons of positive samples were purified using a MultiScreen $PCR_{\mu 96}$ plate (EMD Millipore, Darmstadt, Germany) according to the manufacturer's instructions. The sequencing PCR assay was done using a BigDye Terminator v 1.1 cycle sequencing kit (Applied Biosystems, Zug, Switzerland) according to the manufacturer's instructions and was performed with specifically designed inner primers panFseq (5′-CCAACACTGGGACTGAGA-3′) and panRseq (5′-GCCGGTGCTTCTTTAC-3′) (29). Amplification was performed after an initial denaturation at 96°C for 1 min followed by 25 cycles using a modified program (96°C for 10 s, 60°C for 2 min). The purification of sequencing PCR products was done using a BigDye Xterminator kit (Life Technologies) according to the manufacturer's recommendation, and sequencing was performed in a 3130xL genetic analyzer (Applied Biosystems). The sequences are listed in Table S1 in the supplemental material.

Estimation of individual-level *Chlamydiales* DNA prevalence in ticks. Maximum likelihood estimates of individual *Chlamydiales* DNA prevalence in ticks were calculated by using an online method and tools of the Australian Biosecurity Cooperative Research Centre for Emerging Infectious Diseases (http://www.abcrc.org.au). The method is based on a generalized linear modeling to calculate maximum likelihood estimates of prevalence, taking into account that collected pools were of different sizes and assuming that our test is perfectly sensitive and specific (42). The confidence level was 0.95.

RESULTS

62,889 *Ixodes ricinus* ticks (42,576 nymphs and 20,313 adults) were collected throughout Switzerland and consolidated into 8,534 pools of samples of 1 to 10 arthropods in order to obtain 4,331 pools of nymphs and 4,203 pools of adults. Nucleic acids were extracted automatically, and samples were investigated for the presence of *Chlamydiales* DNA by using a quantitative pan-*Chlamydiales* PCR. Interestingly, results were positive for 543 pools, corresponding to a pool prevalence of 6.4% (543/8,534). Positive samples were equally distributed between the pools composed of nymphs, with a prevalence of 6.9% (298/4,331), and the pools composed of adults, with a prevalence of 5.8% (245/4,203) (Table 1). This prevalence per pool corresponds to the presence of at least one *Chlamydiales* DNA within each positive pool and represents a theoretical prevalence obtained independently of the number of infected ticks in each positive pool. Taking into account the different parameters, which are the pool size, the number of pools tested, and the number of positive pools, the estimated individual prevalence per individual tick was dramatically lower than the maximum prevalence per pool, being 0.89% (1.24% for the adults and 0.72% for the nymphs, respectively). Thanks to the availability of a large number of pools of different sizes, the estimated prevalence was very close to the minimum infection rate of 0.86%, assuming that only one tick in each positive pool was infected, while the pool prevalence was very close to the maximum infection rate of 6.6%, assuming that all ticks in

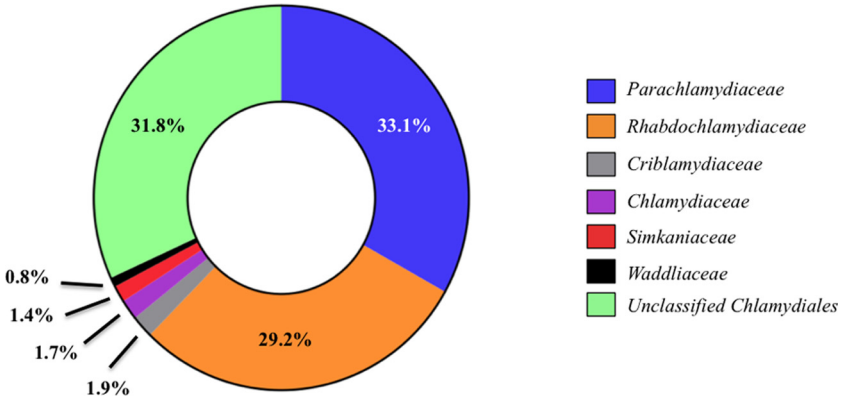

FIG 1 Family distributions of the sequenced pan-*Chlamydiales* qPCR-positive samples. The proportion of each *Chlamydiales* family-level lineage among the sequenced samples was calculated. Please note that the *Rhabdochlamydiaceae* family and the *Parachlamydiaceae* family represented two-thirds of the sequenced samples.

each positive pool were infected (Table 1). Sequencing was performed for all 543 of the positive pools, and, despite uninterpretable sequencing for 184 samples (33.8%), 359 sequences (66.2%) were obtained and submitted to a BLAST analysis in order to compare them with reference *Chlamydiales* 16S rRNA gene sequences. Thus, 10 sequences exhibited best BLAST hit identities ranging from 86% to 89.7%, which correspond to a new family-level lineage according to the taxonomy cutoff defined by Everett et al. (2). Moreover, 181 samples exhibited identity between 90% and 95%, 65 samples identity between 95% and 97%, and 99 samples identity above 97%. According to the cutoff currently used for the taxonomy of the *Chlamydiales* (2, 3), these samples represented, respectively, 181 putative new genera, 65 putative new species, and, finally, 99 putative new strains (see Fig. S1 in the supplemental material).

Best BLAST hits with more than 90% identity have been obtained for 349 sequences, which were taxonomically assigned at the family-level lineage (Fig. 1). *Parachlamydiaceae* and *Rhabdochlamydiaceae* were the most commonly documented families within the order *Chlamydiales*. Indeed, 33.1% (119/359) of the sequenced samples belonged to the *Parachlamydiaceae* family, and 29.2% (105/359) belonged to the *Rhabdochlamydiaceae* family, whereas 31.8% (114/359) corresponded to DNA sequences exhibiting the highest homology with unclassified *Chlamydiales* bacteria. The remaining sequences corresponded to *Criblamydiaceae*, *Chlamydiaceae*, *Simkaniaceae*, and *Waddliaceae* families, but with low rates of 1.9% (7/359), 1.7% (6/359), 1.4% (5/359), and 0.8% (3/359), respectively (Fig. 1). Thus, DNA belonging to 6 of the 9 *Chlamydiales* families could be identified. The distribution of these *Chlamydiales* families in ticks was rather homogenous throughout Switzerland, except for *Parachlamydiaceae*, which were mainly identified in northwest Switzerland (Fig. 2).

Among the 119 sequences belonging to the *Parachlamydiaceae* family, 4 genera were represented, with 23 sequences belonging to the genus *Parachlamydia*, 13 with those belonging to the genus *Neochlamydia*, and 9 each with those belonging to the genera *Mesochlamydia* and *Protochlamydia*. The remaining 65 sequences could not be classified at the genus level. The *Rhabdochlamydiaceae* family was nearly as thoroughly represented as the *Parachlamydiaceae* family but exhibited low diversity, since *Rhabdochlamydia* was almost the only genus represented (102/105), 3 sequences being not classified. Families that were less prevalent all exhibited low diversity, since only 3 best BLAST hits were obtained for the genus *Estrella* within the *Criblamydiaceae* family, whereas 3 and 1 best BLAST hits were obtained for the genera *Simkania* and *Fritschea*, respectively, within the *Simkaniaceae* family (Table 2).

Interestingly, among the 543 pools positive for the pan-*Chlamydiales* qPCR, huge differences between samples were observed in terms of DNA quantity. Indeed, the DNA quantity ranged from a very high DNA copy number ($>1 \times 10^6$ copies/μl) to a very low DNA copy number (<10 copies/μl). Nevertheless, more than half of the samples exhibited a DNA copy number of ≥20 copies/μl. Thus, the quantity of *Parachlamydiaceae* DNA was about 300 DNA copies/μl, with no extreme values. In contrast, *Rhabdochlamydiaceae* DNA was on average seen at 20,000 copies/μl, with a large distribution ranging from 10 copies/μl to 8×10^6 copies/μl. For the other families, the quantity of DNA was on average about 20 copies/μl for the *Criblamydiaceae* and the *Chlamydiaceae*, while it was about 100 copies/μl for the *Waddliaceae* and the *Simkaniaceae* (Fig. 3).

DISCUSSION

The biodiversity of *Chlamydiales* is considered to be largely underestimated (15). Moreover, *Chlamydia* are well-established pathogenic bacteria, and growing evidence supports the idea of a role of several *Chlamydia*-related bacterial species as human or animal pathogens (4, 8). However, virtually nothing is known about the ecological niches, the reservoirs, and the interspecies transmission of these bacteria. Arthropods, and particularly ticks, are well known as vectors of viruses and bacteria, and *Chlamydiales* DNA was recovered from tick samples collected in a small rural area in Switzerland (17). In the present work, a large-scale study was performed to screen more than 62,000 *Ixodes ricinus* ticks collected throughout Switzerland for the presence of *Chlamydiales* bacteria. Since the ticks were pooled, a maximum prevalence was calculated, based on the assumption that all ticks of the same positive pool are positive. Thus, the pool prevalence of *Chlamydiales* DNA within *Ixodes ricinus* ticks in Switzerland is 6.4% (542/8,534), with no significant difference between the prevalence of 6.9% (297/4,331) calculated for the pools of adults and the prevalence of 5.8% (245/4,203) calculated for the pools of nymphs. This prevalence per pool is about 4 times lower than the prevalence of 28% observed in the previous study performed in

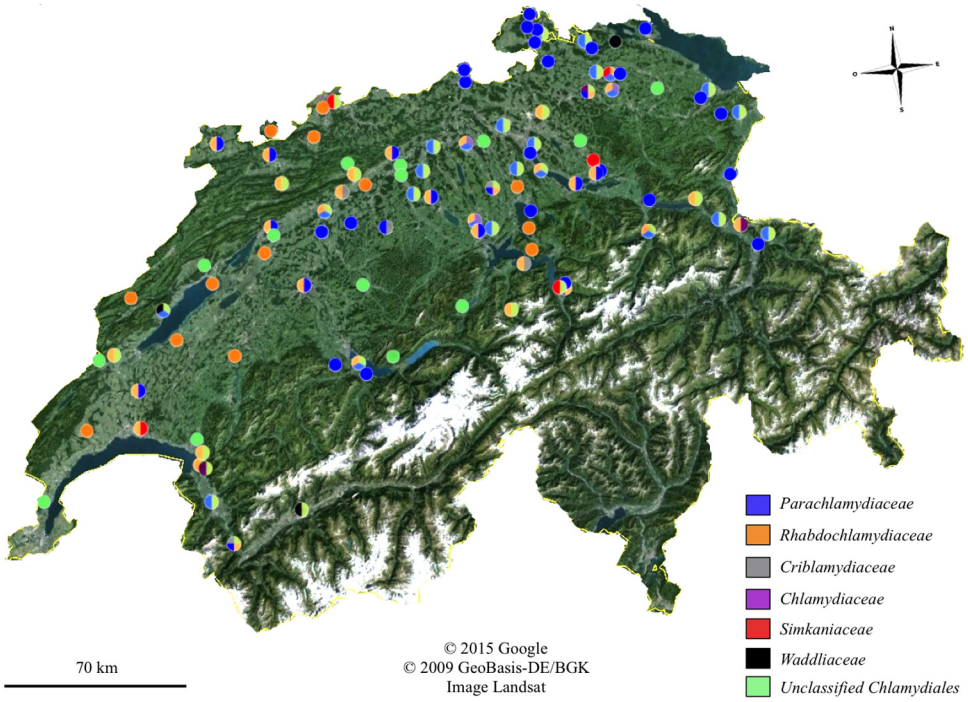

FIG 2 Distribution of DNAs of the *Chlamydiales* families in *Ixodes ricinus* ticks in Switzerland. Among the 543 pools of samples that gave positive results in the pan-*Chlamydiales* qPCR, DNAs from 359 were successfully sequenced. *Chlamydiales* bacterial DNA was recovered from ticks collected in 107 of the 172 collection sites. DNAs of up to 4 *Chlamydiales* families were identified in each area. The geographical distribution of DNAs of *Chlamydiales* families was rather homogenous.

Rarogne (Valais) (17). Moreover, no positive pool was found among the 67 pools collected in the same region (Rarogne) in the present work, suggesting that prevalence can change over time.

The assessed prevalence in individual ticks of 0.89% is similar to the observed prevalence of other bacteria (*Babesia*, *Anaplasma*, *Francisella*, and *Rickettsia*) and viruses (tick-borne encephalitis virus) within ticks in Europe (28, 30–32), with major variations depending on the latitude and the altitude of the collection sites, as well as on the period of collection. It is noteworthy that, in this study, the estimated prevalence in individual ticks is nearly twice as high for the adult ticks (1.24%) as it is for the nymphs (0.72%). A higher prevalence in adult ticks makes sense, assuming a transstadial transmission of *Chlamydiales*, since the developmental cycle of the ticks is sequential, with the nymph stage preceding the adult stage. Moreover, the increased prevalence in adult ticks might also be directly related to blood meals and thus to increased exposure of adult ticks to infected animal hosts. However, transovarian and transstadial transmission would explain the relatively high prevalence already observed in nymphs.

TABLE 2 Sequencing results of the pan-*Chlamydiales* qPCR-positive samples[a]

Family-level lineage	Best BLAST hit(s) at the genus level (*n*)
Parachlamydiaceae	*Parachlamydia* (23), *Neochlamydia* (13), *Mesochlamydia* (9), *Protochlamydia* (9), ND[b] (65)
Rhabdochlamydiaceae	*Rhabdochlamydia* (102), ND (3)
Criblamydiaceae	*Estrella* (3), ND (4)
Chlamydiaceae	ND (6)
Simkaniaceae	*Simkania* (3), *Fritschea* (1), ND (1)
Waddliaceae	ND (3)
Unclassified *Chlamydiales*	

[a] For 359 of the 543 positive samples that have been successfully sequenced, we could establish a taxonomic classification by comparison of the best BLAST hit with reference 16S rRNA gene sequences.
[b] ND, classification at the genus level was not determined.

To obtain more information about the biodiversity of *Chlamydiales* DNA detected in tick samples, all 542 of the positive samples were sequenced. Unfortunately, sequencing failed for 184 samples, representing about one-third of the positive samples. This high rate of sequencing failure was in the majority due to the presence of DNA of *Chlamydiales* originating from different species-level lineages and resulting in sequencing data that were not interpretable. Nevertheless, 359 samples were sequenced and compared to already-known sequenced *Chlamydiales* 16S rRNA genes.

In fact, the sequenced pan-*Chlamydiales* PCR products were short sequences of about 200 bp of the 16S rRNA gene, allowing lineage classification only at the family and genus levels. Even if the targeted region is highly variable (29), and even if it was already proven that short sequences can provide confident classification at the genus-level lineage (33), identification will need to be completed by targeting genes that are known to be more discriminating (34) in order to obtain precise and definitive lineage classification at the genus and species levels. Thus, among the 359 sequenced samples, 119 *Parachlamydiaceae*, 105 *Rhabdochlamydiaceae*, 7 *Criblamydiaceae*, 6 *Chlamydiaceae*, 5 *Simkaniaceae*, 3 *Waddliaceae*, and 114 unclassified *Chlamydiales* have been identified. As already described in a recent study (17), *Parachlamydiaceae* and *Rhabdochlamydiaceae* seem to be the most widely represented *Chlamydiales* bacteria within ticks in Switzerland. Interestingly, only 4 sequences exhibiting 100% identity with a se-

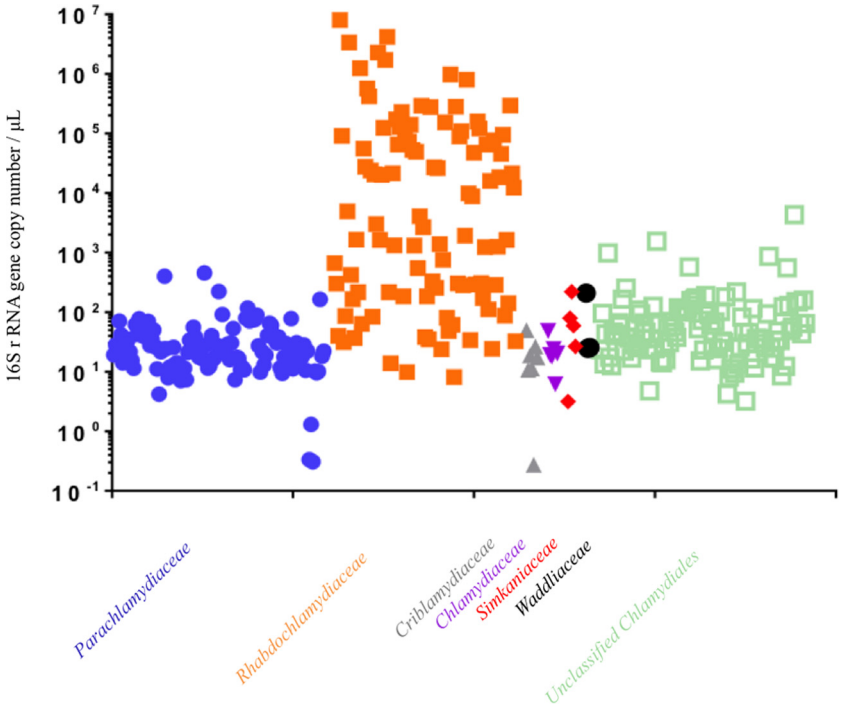

FIG 3 *Chlamydiales* DNA quantity within sequenced samples. The number of 16S rRNA gene copies per microliter in each of the 359 sequenced samples was calculated by comparison with a reference sample.

quence already obtained in our laboratory were observed. All 4 sequences shared 100% identity with "*Candidatus* Rhabdochlamydia crassificans" strain CRIB01. These 4 DNAs were extracted from ticks collected in 4 different cantons, highlighting the widespread scattering of these bacteria within ticks in Switzerland. *Rhabdochlamydiaceae* have been already associated with arthropods (24–26), in contrast to the *Parachlamydiaceae*, exhibiting a symbiotic association mainly with amoebae. Since *Parachlamydiaceae* are considered emerging pathogens (35), this high prevalence of *Parachlamydiaceae* DNA within ticks in Switzerland may be of medical importance. Until now, with the exception of a small study performed in neonates (36), no clear pathogenic role was attributed to *Rhabdochlamydiaceae* bacteria, mainly due to the nearly complete absence of diagnostic tools and to the difficulty encountered in attempts to grow *Rhabdochlamydiaceae*. However, the fact that *Rhabdochlamydiaceae* DNAs are present in huge amounts within *Ixodes ricinus* ticks should trigger studies aiming at investigating the prevalence of these *Chlamydiales* bacteria within wild and farm animals, as well as the prevalence in humans with and without a history of tick bites. Several previous studies suggested a possible transmission of bacteria of the *Chlamydiales* order to humans and animals through ticks (22, 23, 37). This report provides a supplementary hint supporting these observations, and further work will be necessary to increase our knowledge about transmission of *Chlamydiales* bacteria by ticks. Furthermore, even if the geographical distribution seems to be relatively homogenous, further work should focus on the geographical and environmental features associated with the presence of *Chlamydiales* bacteria.

In terms of the biodiversity of the *Chlamydiales* bacteria, the screening provided 359 sequences. It represents well the still-unknown extent of *Chlamydiales* diversity, since 10 of these sequences could be assimilated to potential new families, 181 to potential new genera, and 65 to potential new species.

In summary, high prevalence and biodiversity of DNAs of *Chlamydiales* bacteria within *Ixodes ricinus* ticks collected in Switzerland are reported. Even if the transmission from ticks to birds and mammals deserves to be investigated, ticks should be considered potential new vectors and reservoirs for *Chlamydiales* bacteria. By using tick cell lines and other innovative culture methods (19), further work should be begun to isolate new species from tick samples, as has already been done to isolate *Anaplasma* and *Rickettsia* species (38–41). Moreover, since huge quantities of *Chlamydiales* DNA have been obtained from some ticks, it should be a unique opportunity to obtain genomic data for some still-uncultivable *Chlamydiales*, including new family-level lineages, and in this way increase our knowledge of *Chlamydiales* biology, diversity, and ecology.

ACKNOWLEDGMENTS

This work was supported by a grant from the Public Health Office. Research by the group of G.G. is also supported by SNSF grant no. 141050, which covers the salary of L.P.

The Spiez laboratory and the Swiss Army performed the collection of the ticks.

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